



## Mini-Review

# Applications of Solids NMR to the Analysis of Insect Sclerotized Structures

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This article reviews the solids NMR research conducted on insect sclerotized structures in the last 10 years and previews some of the experiments that will be conducted in the future. Solids NMR has been used as a noninvasive approach to investigate the chemical compositions of, and some covalent interactions that occur in, several types of sclerotized structures that are otherwise highly intractable to conventional chemical analyses. Sclerotization is a complex process used by insects to confer stability and mechanical versatility to their cuticular exoskeletons and certain other proteinaceous structures. Samples analyzed include cuticular exoskeletons, egg cases, egg shells, cocoons and peritrophic membranes. Cross polarization, dipolar decoupling, magic angle spinning, magnetization dephasing, and isotropic enrichment were used to obtain high resolution spectra that provide information about the types and relative concentrations of carbon atoms as well as internuclear distances and covalent bonds between carbon and nitrogen atoms. Relative amounts of protein, chitin, catechols, lipids, pigment, and oxalate were estimated. Covalent interactions between protein nitrogens and catechol carbons were detected in the stiff brown pupal cuticle of the tobacco hornworm, *Manduca sexta*. The results of these solids NMR studies support the hypothesis that sclerotization of insect structures occurs primarily when quinones derived from *N*-acylcatecholamines form cross-links and adducts with functional groups of proteins deposited in the structures. Future applications of solids NMR will utilize advanced techniques for further probing the covalent interactions of  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{17}\text{O}$ -labeled catechols, chitin and protein in sclerotized structures.

NMR   Cuticle   Exoskeleton   Sclerotization   Cross-links   Pigmentation   Chitin   Protein   Catechols  
Oxalate   Melanin

### INTRODUCTION

Nuclear magnetic resonance (NMR) techniques were developed originally to investigate nuclear properties of atoms (Rabi *et al.*, 1939; Becker, 1993). Eventually, NMR was found to be a powerful tool for solving problems in structural chemistry, biochemistry, and, most recently, medical imaging. In the past 20 years with the development of line-narrowing and gradient imaging methods, NMR spectroscopy has blossomed into a

major technique in solid-state materials science and now provides a noninvasive approach to investigate detailed structures in heterogeneous materials such as biopolymeric composites. Since the mid 1980s, we have used solid-state NMR to determine the chemical compositions of cuticles, egg cases, egg shells, cocoons, and peritrophic membranes and also to probe the types of covalent interactions that arise when some of these materials are assembled and stabilized. Because of the intractable nature of insect sclerotized structures, there is very little quantitative data available about chemical composition. This article reviews some of the progress made when solids NMR was applied to problems in insect biochemistry and also discusses some developments that we anticipate in the future. It focuses on compositional and developmental information sought by these interested in insect biochemistry, rather than details of the NMR methods. Previously, the use of solution and solids NMR in studies of insect metabolism was reviewed by Thompson (1990).

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## SOLIDS NMR METHODOLOGY

NMR measures the radio frequencies emitted by and the rates of realignment of nuclear spins in a magnetic field after atomic nuclei absorb energy from radio frequency pulses. The single-resonance, natural abundance  $^{13}\text{C}$  NMR spectrum of a solid usually consists of a single, broad, featureless line, with a width of the order of 20 kHz. The major source of line broadening in this situation is the static dipolar interaction between carbons and nearby protons. These protons include covalently bonded methine, methylene, or methyl protons, together with more distant, indirectly bonded protons. Dipolar interactions depend upon the orientations of internuclear vectors with the applied magnetic field. In a crystal powder or an amorphous material, all orientations occur, resulting in a broad distribution of dipolar splittings.

This dipolar broadening can be removed in a straightforward way. If the  $^{13}\text{C}$  resonance is observed in the presence of a strong radio frequency field (rf) at the Larmor frequency of the protons, the protons undergo rapid transitions of spin flips which cause the time-averaged dipolar field generated by the protons at the carbon nucleus to disappear. The entire process is known as dipolar decoupling, and is analogous to the more familiar scalar decoupling used to remove spin-spin splitting from high resolution  $^{13}\text{C}$  NMR of liquids. The only difference is the strength of the rf fields used in the two experiments. Dipolar decoupling requires rf fields greater than the local fields experienced by the protons arising from  $^1\text{H}$ - $^1\text{H}$  and  $^1\text{H}$ - $^{13}\text{C}$  interactions. For a typical solid this might require an rf field of 40 kHz, or about 10 times as large as is necessary to perform ordinary scalar decoupling.

With dipolar decoupling, the  $^{13}\text{C}$  NMR spectrum of a solid begins to show signs of improved resolution. A typical spectrum now has a width of the order of 15 kHz at a  $^{13}\text{C}$  Larmor frequency of 50 MHz with a few features clearly evident. The spectrum is not, however, of liquid-like high resolution quality. The remaining broadening is due to chemical shift anisotropy (CSA). The magnetic field at a carbon nucleus depends upon the shielding or screening afforded by the surrounding electron density. In general, the surrounding electron density is not symmetric. Thus, the chemical shift of, say, a carbonyl carbon in an ester group, depends upon whether the C—O carbonyl is lined along the magnetic field, or happens to be perpendicular to it, or is in some other orientation. In a liquid with rapid molecular motion, only an average, or isotropic chemical shift is observed. In a single crystal, a single chemical shift may be observed, but its value depends upon the orientation of the crystal relative to the magnetic field. In an amorphous solid or crystal powder, on the other hand, a complicated lineshape is observed that arises from the sum of all possible chemical shifts.

The line broadening effect of the CSA is now apparent. For a typical solid with a variety of chemically

different carbons, the  $^{13}\text{C}$  NMR spectrum is a sum of different CSA patterns, having somewhat different shapes, and most importantly, having different isotropic centers. Overlapping CSA patterns for carbonyl carbons, aromatic carbons, and aliphatic carbons destroy the resolution one had hoped to gain from dipolar decoupling.

Fortunately, there is a method to regain the lost resolution. The broadening arises from restrictions placed on molecular motion in the solid. Experimentally, we can supply a kind of molecular motion ourselves. We can do this by mechanically rotating the sample about the diagonal of a cube whose edges are the rectilinear coordinate system defined by the applied magnetic field. The rotation axis is at half the tetrahedral angle relative to the applied field, the so-called "magic angle" of  $54.7^\circ$ . The mechanical rotation interchanges axes and internuclear directions relative to the magnetic field and therefore has many of the same averaging properties as isotropic motion. Fast magic angle spinning is achieved using the same kind of gas-supported bearings that are used in ultracentrifuges. The combination of dipolar decoupling and fast magic angle spinning affords resolution that is usually only limited by variations of bulk susceptibility within the sample, typically 0.5 ppm.

Solids NMR experiments combine cross polarization for sensitivity enhancement of the signal to noise ratio with dipolar decoupling to remove dipolar interactions from protons and magic angle spinning (MAS) for high resolution of chemical shifts (Hartman and Hahn, 1962; Lurie and Slichter, 1964; McArthur *et al.*, 1969; Pines *et al.*, 1973; Andrew, 1971). Assignments of carbon chemical shifts are made by comparison with literature

TABLE 1. Chemical shift assignments of resonances in the CPMAS  $^{13}\text{C}$  NMR spectra of insect support structures

Resonance no.	Values (ppm)*	Assignment
1	172	Carbonyl carbons of chitin, protein, lipid, and catechol acyl groups
2	170	Carbonyl carbons of oxalate
3	155	Phenoxy carbon of tyrosine, guanidino carbons in arginine
4	144	Phenoxy carbons of catechols
5	129	Aromatic carbons
6	116	Tyrosine carbons 3 and 5, imidazole carbon 4, catechol carbons 2 and 5
7	104	GlcNAc carbon 1
8	82	GlcNAc carbon 4
9	75	GlcNAc carbon 5
10	72	GlcNAc carbon 3
11	60	GlcNAc carbon 6, amino acid $\alpha$ -carbons
12	55	GlcNAc carbon 2, amino acid $\alpha$ -carbons
13	44	Aliphatic carbons of amino acids, catechols, and lipids
14	33	Aliphatic carbons of amino acids, catechols, and lipids
15	23	Methyl carbons of chitin, protein, lipid, and catechol acyl groups; amino acid methylene carbons
16	19	Methyl carbons of amino acids and lipids

\* $\delta$ -Values relative to external TMS reference.

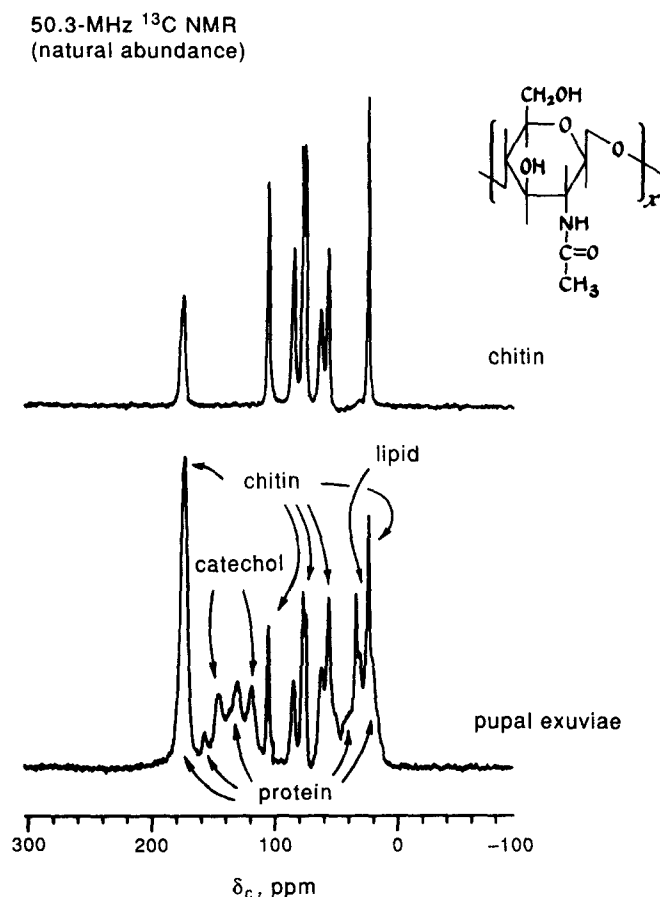


FIGURE 1. Cross polarization magic angle spinning 50 MHz  $^{13}\text{C}$  NMR of *M. sexta* chitin (top) and pupal exuviae (bottom). See Table 1 for chemical shift assignments.

values or chemical shifts of standard compounds. Table 1 lists the chemical shift assignments in the  $^{13}\text{C}$  CPMAS NMR spectra of insect support structures. For example, carbon chemical shifts for insect chitin are assigned by comparison to carbon solution or solids NMR spectra of 2-acetamido-2-deoxyglucopyranoside and crab chitin (Fig. 1; Fukamizo *et al.*, 1986). Chitin, protein, catechol and lipid carbon signals dominate the natural abundance  $^{13}\text{C}$  solid state NMR spectrum of *M. sexta* pupal exuviae that are composed of approx 30% chitin. Absolute or relative concentrations of carbons can be determined by extrapolation of signal intensity to zero contact time and comparison with the signal from an external standard compound. Difference spectra from single and double cross-polarization (abbreviated CP and DCP, respectively) experiments allow measurement of heteronuclear coupling between two stable isotopes that are within approx 2 Å of each other.

Rotational echo double resonance (REDOR), a relatively recent magic angle spinning solid-state NMR technique, is orders of magnitude more sensitive than DCP in measuring long-range heteronuclear interactions up to about 5 Å for  $^{13}\text{C}$ – $^{15}\text{N}$ , 8 Å for  $^{13}\text{C}$ – $^{31}\text{P}$  and 12 Å for  $^{13}\text{C}$ – $^{19}\text{F}$ . For REDOR analysis, magnetization on one rare spin radio frequency channel is dephased by rotor-synchronized pulses on a second isotope channel; interactions with protons are suppressed by dipolar

decoupling. The extent of dephasing has a simple relation to the strength of the dipolar coupling and the internuclear distance. REDOR provides a direct measure of heteronuclear dipolar coupling between isolated pairs of labeled nuclei. In a solid with a  $^{13}\text{C}$ – $^{15}\text{N}$  labeled pair, for example, the  $^{13}\text{C}$  rotational echoes that form each rotor period following a  $^1\text{H}$ – $^{13}\text{C}$  cross polarization transfer can be prevented from reaching full intensity by insertion of an  $^{15}\text{N}$   $\pi$  pulse each half rotor period. The REDOR difference (the difference between a  $^{13}\text{C}$  NMR spectrum obtained under these conditions and one obtained with no  $^{15}\text{N}$   $\pi$  pulses) has a strong dependence on the  $^{13}\text{C}$ – $^{15}\text{N}$  dipolar coupling and hence the  $^{13}\text{C}$ – $^{15}\text{N}$  internuclear distance.

REDOR is described as double resonance even though three radio frequencies (typically  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$ ) are used because the protons are removed from the important evolution part of the experiment by resonant decoupling. The dephasing of magnetization in REDOR arises from a local dipolar  $^{13}\text{C}$ – $^{15}\text{N}$  field gradient and involves no polarization transfer. REDOR has no dependence on  $^{13}\text{C}$  or  $^{15}\text{N}$  chemical shift tensors and does not require resolution of a  $^{13}\text{C}$ – $^{15}\text{N}$  coupling in the chemical shift dimension.

Another technique that enables high resolution NMR analysis of specific atoms in compounds is selective isotopic enrichment, which helps to filter out the background of both isotopic natural abundance and similar chemical shifts that occur in rather complex molecular assemblies or mixtures. We have used all of these methods to examine the chemical compositions and structures of a variety of intractable solid materials from insects, all of them not easily amenable to analysis by conventional solution-based techniques. We have employed MAS solids NMR for measuring distances between pairs of spin-1/2 heteronuclei. In particular, our method of choice focuses on determining specific internuclear separations between dilute  $^{13}\text{C}$ ,  $^{15}\text{N}$  spin pairs that can be incorporated into samples by isotopic enrichment. The low natural abundance of  $^{13}\text{C}$  and  $^{15}\text{N}$  (1.11 and 0.37 atom%, respectively) makes these spins ideal candidates for site-specific isotopic labeling. Internuclear distances between spin pairs can be determined from the  $1/r^3$  distance dependence of the dipolar interaction.

Solids NMR experiments are not inexpensive. Major costs are incurred from construction of spectrometers and probes, and, to a lesser degree, the synthesis or purchase of compounds with specific atoms enriched with NMR-active nuclei. Although there are a number of commercial instruments scattered across the country, they are primarily solution-based instruments adapted for solid samples, and therefore were not designed solely for the analysis of solids, which limits their performance. Also, a rather large sample (>100 mg) is needed for solids NMR analysis, and in the case of labeling, a biological specimen that incorporates several micromoles of label into appropriate chemical structures. For most of our experiments, the tobacco hornworm, *Manduca sexta*, was a practical model. A mature hornworm

can attain a mass of >10 g and form a pupal exoskeleton with a mass of approx 100 mg.

### SOLIDS NMR APPLICATIONS

In 1976 when J. Schaefer and E. O. Stejskal were employed at Monsanto Co. in St Louis, they were the first researchers to obtain spectra of good resolution from completely solid amorphous samples (Schaefer and Stejskal, 1976). Eight years later, Martin Peter's group at the University of Bonn in Germany first used solids NMR to study the compositions of moth, locust, and cockroach cuticles (Peter *et al.*, 1984). Similar natural abundance  $^{13}\text{C}$  NMR spectra were collected for pupal cases of *M. sexta*; exuviae of the migratory locust, *Locusta migratoria*; and exuviae of the cockroach, *Blaberus giganteus*, which generally indicated comparable compositions of protein, chitin, and catechols in most of these structures. Those results were interpreted to be consistent with the hypothesis that the sclerotization of cuticle occurs by the denaturation of structural proteins by polyphenolic compounds, but the actual mechanism of sclerotization was not determined.

Subsequently, we reported the results of a collaborative study on the composition and several heteronuclear interactions of pupal and moth cuticle of *M. sexta* (Schaefer *et al.*, 1987). Selective  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopic enrichment of pupal cuticle by injection of appropriately labeled precursor amino acids and catecholamines enabled the detection of covalent linkages between ring nitrogens of protein histidyl residues and ring carbons of the catecholamine, dopamine, which is a precursor of quinonoid tanning agents. Those results support the hypothesis that the stiffening of insect cuticle during sclerotization is correlated with the deposition of protein and chitin polymers and their cross-linking by quinonoid derivatives of catecholamines. Since then, many other types of insect support structures have been examined using solids NMR to gain insight into the mechanisms whereby insects assemble and stabilize these materials. The results of those studies are discussed below.

Williams *et al.* (1988) at Texas A&M University compared  $^{13}\text{C}$  NMR spectra of *Heliothis virescens* pupal cuticles dissected from  $\beta$ - $^{13}\text{C}$  labeled tyrosine-injected and control insects. Incorporation of the  $^{13}\text{C}$ -labeled tyrosine into the cuticle had little effect on the chemical shift of the labeled atom, indicating that the side chain  $\beta$ -carbon was not modified heavily during cuticle morphogenesis. The authors hypothesized that the  $\beta$ -carbon of tyrosine and/or its metabolites are not involved in protein cross-linking reactions during *Heliothis* cuticle formation.

#### Chemical compositions

Samples analyzed for organic chemical (carbon) composition include insect chitin, egg shells, cocoons, egg cases, peritrophic membrane, and cuticles. These ma-

terials often contain a few percent of water and ash, both of which are not quantifiable by NMR analysis, but instead are determined by gravimetric analysis.  $^{13}\text{C}$  NMR was used for carbon compositional analysis, and natural abundance  $^{13}\text{C}$ -CPMAS spectra were obtained on samples that were cleaned of adhering tissues, washed with either deionized water or a dilute detergent solution, frozen, lyophilized, and finally ground into a powder with a particle size of approx 40 mesh or smaller. The samples analyzed range in composition from relatively homogeneous to highly heterogeneous. We begin this discussion with one of the most homogeneous materials, chitin, and end with one of the most heterogeneous structures, cuticle.

#### Chitin

Chitin extracted from tobacco hornworm larval cuticle by exhaustive boiling in 1 M sodium hydroxide was one of the first insect materials examined by solids NMR (Peter *et al.*, 1984; Fukamizo *et al.*, 1986). Chitin is the major polysaccharide in cuticle and is composed of  $\beta(1 \rightarrow 4)$  linked 2-deoxy-2-acetamido-D-glucopyranosyl residues. The hornworm preparation is >99% organically homogeneous, with the remainder being residual amino acids (Fig. 2). Little or no glucosamine/chitosan is present since the degree of *N*-acylation in acetic anhydride-treated and untreated samples is comparable, as inferred from zero contact time extrapolated intensities of the methyl and other carbon signals. The relative carbon intensities are near theoretical values

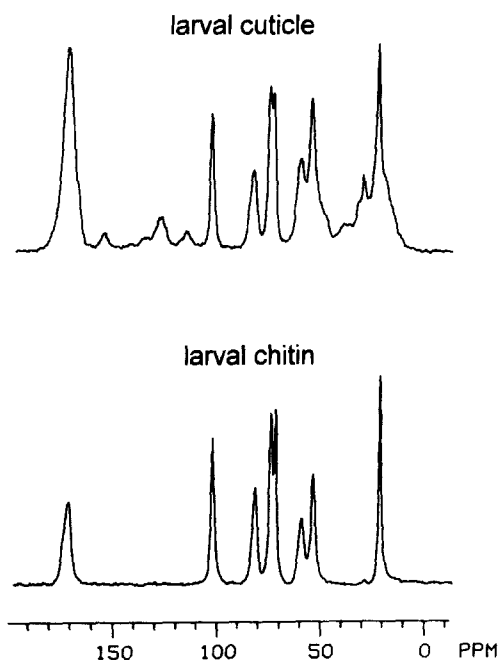


FIGURE 2.  $^{13}\text{C}$  Natural abundance cross-polarization magic angle spinning NMR spectra of *Manduca sexta* larval cuticle (upper spectrum) and larval chitin (lower spectrum). The carbohydrate carbon chemical shift assignments are carbonyl, 172 ppm; C-1, 104 ppm; C-4, 82 ppm; C-5, 75 ppm; C-3, 72 ppm; C-6, 60 ppm; and methyl, 23 ppm. See Table 1 for other chemical shift assignments. The scale is in parts per million downfield from tetramethylsilane (TMS) used as an external reference. From Fukamizo *et al.*, 1986.

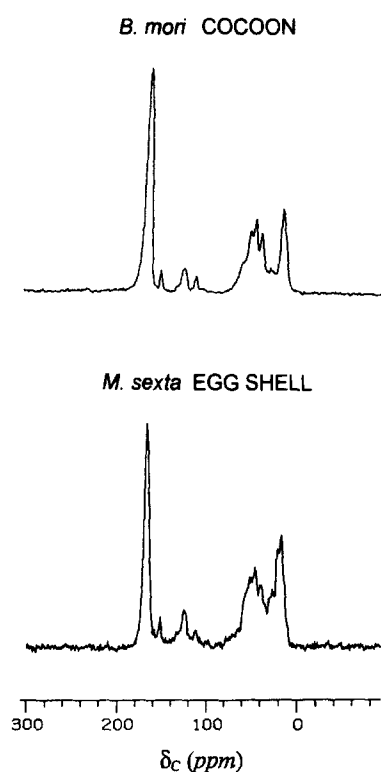


FIGURE 3. Solid state  $^{13}\text{C}$  NMR spectra of *Manduca sexta* egg shells and *Bombyx mori* cocoons. See Table 1 for chemical shift assignments.

for *N*-acetylglucosamine. In the spectrum of intact hornworm larval cuticle, eight of the major resonances observed are primarily due to carbon atoms from chitin (Fig. 2). The rest of the resonances are primarily from protein carbons. Thus, solids NMR demonstrated that the major organic component of hornworm larval cuticle is chitin with a high degree of chemical homogeneity and also that the conditions of hot alkali used to extract the

TABLE 2. Chemical composition of cocoons from four species of silkmoths as determined by solids NMR analysis\*

Species	Relative amount (%)†			
	Protein	Catechol	Water	Ash
<i>Antheraea mylitta</i>	88	1.0	8	3
<i>Hyalophora cecropia</i>	88	0.9	7	4
<i>Antheraea polyphemus</i>	88	0.4	8	4
<i>Bombyx mori</i>	90	0.1	7	3

\*From Kramer *et al.*, 1989.

†Protein and catechol content estimated by  $^{13}\text{C}$  NMR. Water and ash analyzed gravimetrically. Unit = percentage of wet wt.

chitin from the cuticle and digest away the protein do not cause significant deacetylation.

#### Egg shells, cocoons and egg cases

Other structural materials studied using solids NMR, which are relatively homogeneous in organic components, but are primarily protein instead of chitin, included tobacco hornworm egg shells, silkmoth cocoons, and cockroach and mantid egg cases. Proteins are strongly evident in the  $^{13}\text{C}$  NMR spectra of *M. sexta* egg shells and *Bombyx mori* cocoons (Fig. 3), both of which are relatively colorless structures, as evidenced by resonances at 44, 116, 129 and 155 ppm (Table 1, Kramer *et al.*, 1989a). Both materials are composed of >99% protein that may be stabilized by interpeptide cross-links. Moth cocoon silk is made up primarily of two proteins: the thread-like protein, fibroin, and the glue-like protein, sericin (Seifter and Gallop, 1966). Fibroin composition and conformation were analyzed using solids NMR by Fujiwara and Kobayashi (1986). Other types of cocoons from the wild silkmoths, *Antheraea polyphemus*, *A. mylitta* and *Hyalophora cecropia*, are

TABLE 3. Organic composition of egg cases and exuviae from five species of cockroaches as determined by solids NMR analysis\*

Species	Relative amount (%)				
	Protein	Chitin	Catechol	Lipid	Oxalate
<i>Periplaneta americana</i>					
Egg case	87	—	4	1	8
Exuviae	49	38	11	2	—
<i>P. fuliginosa</i>					
Egg case	86	—	6	1	7
<i>Blattella germanica</i>					
Egg case	95	—	3	1	<1
Exuviae					
Wild type	59	30	9	2	—
Yellow	44	41	13	2	—
Orange	46	40	12	2	—
Black	51	38	9	2	—
<i>Blatta orientalis</i>					
Egg case	88	—	4	1	7
<i>Gromphadorhina portentosa</i>					
Exuviae	53	38	8	1	—
<i>Blaberus craniifer</i>					
Exuviae	52	42	5	1	—
<i>Leucophaea maderae</i>					
Exuviae	61	35	4	1	—

\*From Kramer *et al.*, 1991.

## COCKROACH EGG CASES

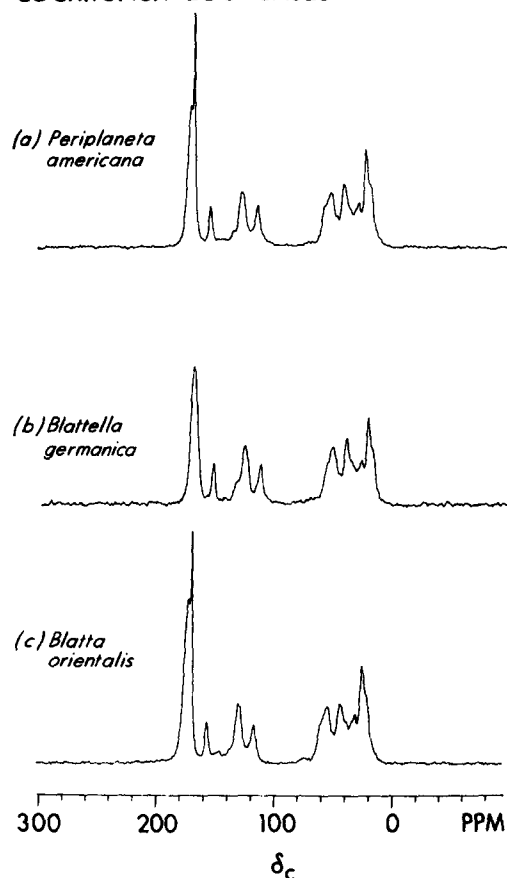


FIGURE 4. Natural abundance CPMAS  $^{13}\text{C}$  NMR spectra of egg cases from (a) *Periplaneta americana*, (b) *Blattella germanica*, and (c) *Blatta orientalis*. From Kramer *et al.*, 1991. See Table 1 for chemical shift assignments.

lightly tanned and, in addition to protein, contain 0.4–1% catecholic compounds (Table 2). These catecholic compounds may add strength to the structures by cementing or cross-linking the silk proteins together.

Egg cases of the praying mantids, *Stagmomantis carolina* and *Tenodera sinensis*, contain 93% protein and 7% phenolics (Kramer *et al.*, 1989a). Cockroach egg cases are similar to mantid egg cases in organic compo-

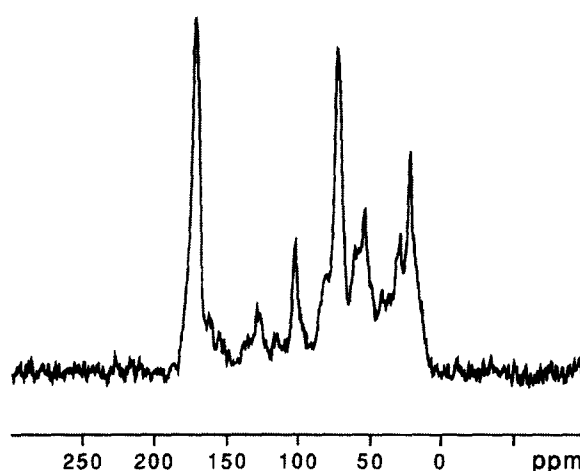
*M. sexta* PERITROPHIC MEMBRANE

FIGURE 5.  $^{13}\text{C}$  NMR spectrum of the peritrophic membrane of the fifth larval instar of *Manduca sexta*. See Table 1 for chemical shift assignments.

sition (Table 3), except that some of the former structures also contain the two-carbon dicarboxylic acid, oxalate, which probably is associated with calcium and may further render the egg case hard and brittle (Kramer *et al.*, 1991). The American and oriental cockroaches deposit egg cases that contain about 7–8% oxalate (170 ppm, Fig. 4), whereas the egg case of the German cockroach has little or no oxalate present.

*Peritrophic membrane*

A structural material secreted by the midgut epithelium that lines and protects the microvilli or brush border of the midgut cells is the peritrophic membrane or matrix (PM). It partitions the lumen into regions between which the transfer of macromolecules, food particles, and microorganisms is limited by the membrane's permeability properties. The PM of the tobacco hornworm is made up primarily of protein (60%) and chitin (40%). As expected, its  $^{13}\text{C}$  NMR spectrum (Fig. 5) resembles a composite spectrum of chitin plus protein.

TABLE 4. Chemical composition of *Manduca sexta* cuticular structures as determined by solids NMR and gravimetric analyses\*

Sample	Water	Protein	Chitin	Catechol	Lipid	Mineral
<i>Larval</i>						
Thorax/abdomen	73	10	14	1	1	1
Head capsule	59	14	12	13	1	1
Mandible	32	24	18	21	3	2
Exuviae	69	15	11	3	1	1
<i>Pupal</i>						
Unsclerotized	79	14	2	1	3	1
Sclerotized	49	19	25	4	2	1
Exuviae	13	31	34	17	4	1
<i>Adult</i>						
Thorax/abdomen	52	28	7	7	5	1
Wing	41	34	12	8	4	1

\*From Schaefer *et al.*, 1987. Unit = percentage of wet weight. Protein, chitin, catechol, and lipid determined by  $^{13}\text{C}$  NMR; water and mineral (ash content) by gravimetric analysis.

### Cuticles

Insect cuticle is a composite material, primarily a polymeric structure of protein and chitin chains with lesser amounts of catechols, lipids, minerals, pigments and water. It has been studied intensively by solids NMR. The compositions of moth, beetle, and cockroach cuticles and some of the changes in composition that occur when the exoskeleton is stabilized have been determined. For example, the compositions of *M. sexta* cuticular structures (Table 4), including those of sclero-

tized larval, pupal, and adult cuticles (Fig. 6), are presented. The peptide backbone  $\alpha$ -carbon signals at 55 ppm and 60 ppm (see Table 1 for chemical shift assignments) are used to estimate general protein levels, whereas signals at 104 ppm (chitin carbon 1) and 82 ppm (chitin carbon 2) are representative of chitin content because they are due to single carbon types; are well resolved from other resonances; and appear as prominent, well-defined signals in the spectral background of whole cuticular material. The aromatic catecholic ring carbon signal at 144 ppm and the methylene carbon

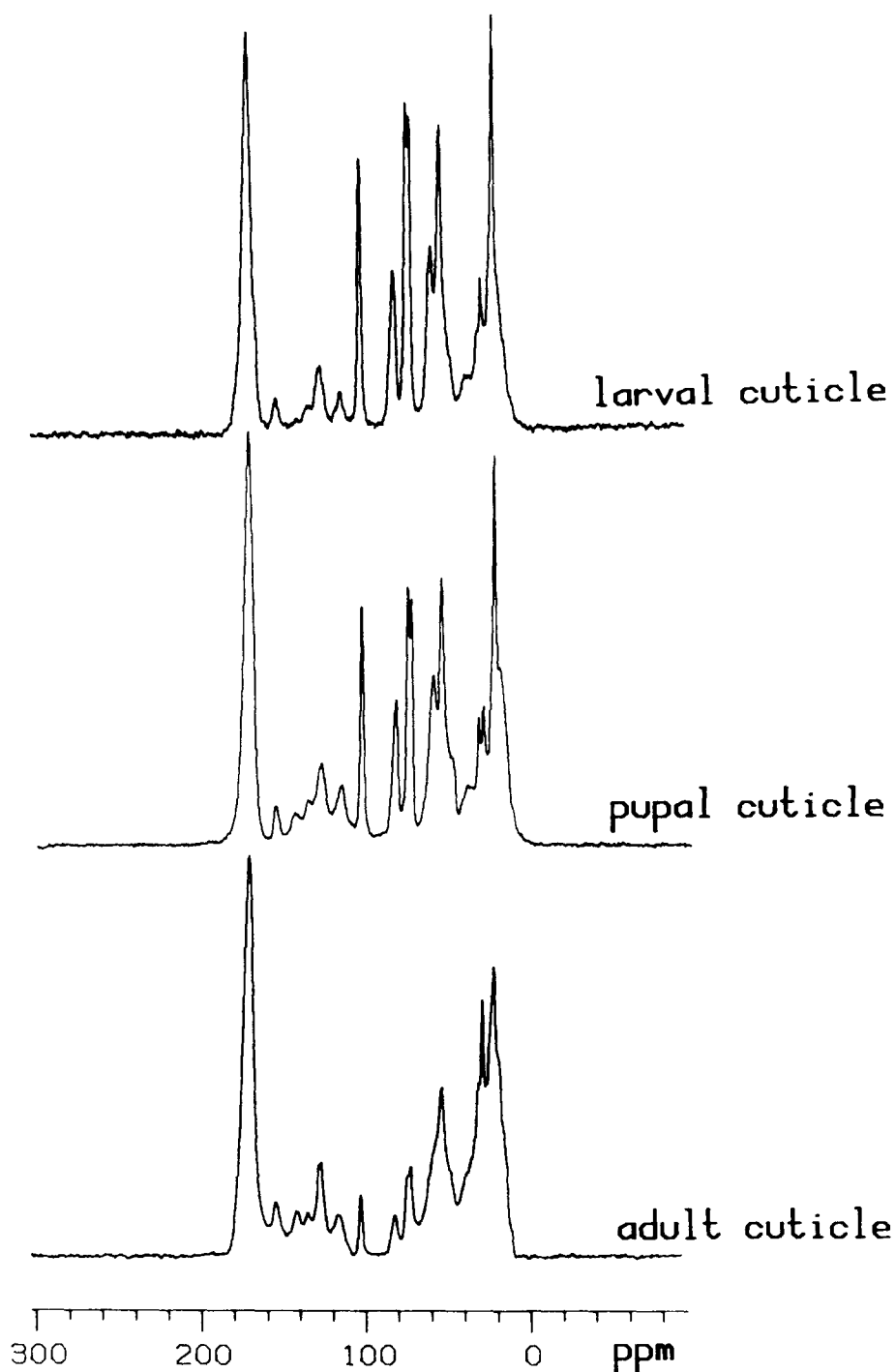


FIGURE 6. <sup>13</sup>C NMR spectra of *Manduca sexta* larval, pupal, and adult cuticles. Cuticles from the head and appendages were not included in the larval and adult samples, and the scales were removed from the adult cuticle. See Table 1 for chemical shift assignments.

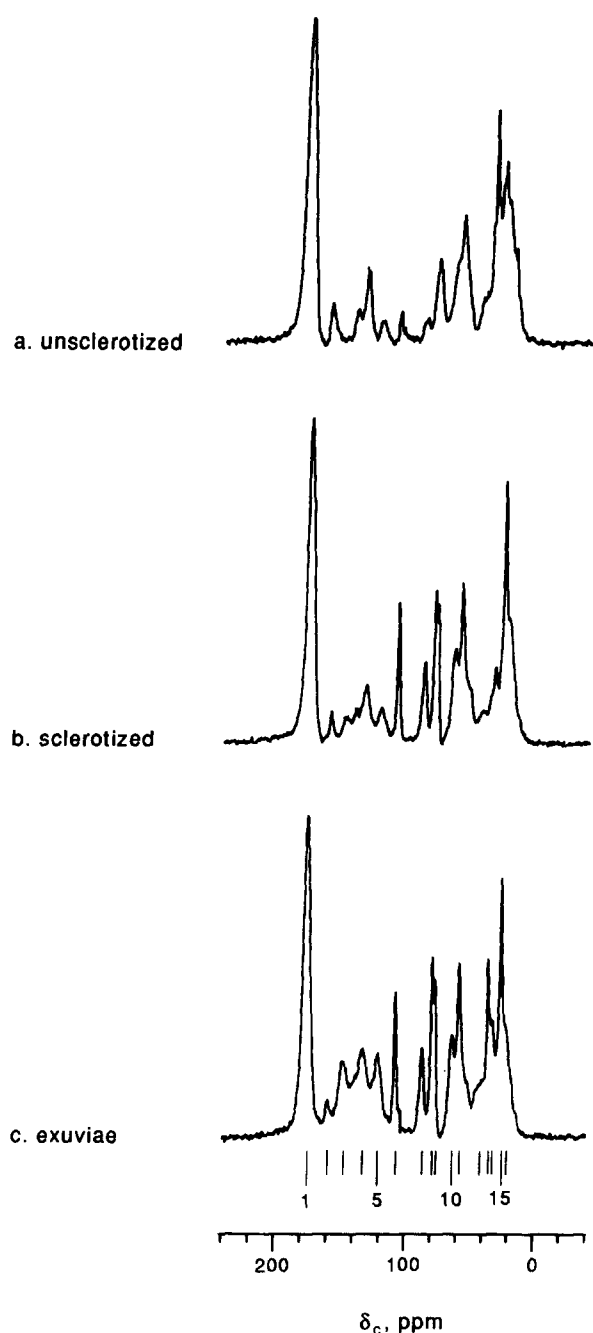


FIGURE 7. CPMAS  $^{13}\text{C}$  NMR spectra of newly ecdysed and relatively unsclerotized *Manduca sexta* pupal cuticle (a), 3-day-old sclerotized pupal cuticle (b), and pupal exuviae consisting primarily of highly sclerotized exocuticle (c). From Schaefer *et al.* (1987). See Table 1 for chemical shift assignments.

signal at 33 ppm are used to estimate catechol and lipid levels, respectively.

The larval cuticle of *M. sexta* is very flexible and largely unpigmented; the pupal cuticle is stiff and brown in coloration; and the adult cuticle, excluding scales, is thin, lightly colored, stiff, and adapted for flight behavior. On a relative concentration basis, adult cuticle exhibits the highest protein content and the lowest chitin content (Table 4). However, larval and pupal cuticles are approximately equal in relative amounts of protein and chitin, with the exception of unsclerotized pupal cuticle.

Chitin content is low relative to protein in pupal cuticle at ecdysis, but becomes equal after sclerotization. Catechols are abundant in pupal and adult abdominal and thoracic cuticles and larval mandible and head capsule cuticles, but are very low in larval abdominal and thoracic cuticles. The major organic difference detected by solids NMR analysis between stiff (pupal, adult, larval head capsule and mandible) and flexible (larval body) cuticular structures of *M. sexta* is the presence of higher levels of catechols in the former.

Changes in chemical composition during sclerotization of the pupal cuticle of *M. sexta* were determined using solids NMR (Schaefer *et al.*, 1987). The unsclerotized cuticle spectrum is composed primarily of protein and lipid carbon signals [Fig. 7(a) and Table 4]. The sharp signal at 33 ppm indicates that a significant amount of lipid is present in untanned cuticle, perhaps as the newly secreted waxy layer of the epicuticle. The unsclerotized pupal cuticle spectrum resembles that of sclerotized adult cuticle, except for the higher levels of catechols indicated by the signal at 144 ppm in the latter (Fig. 6). After sclerotization has occurred, increased relative percentages of chitin and catechols are apparent in contrast to decreased percentages of lipid [(Fig. 7(b))]. The  $^{13}\text{C}$  spectrum of the pupal exuviae, which is the outermost and most heavily sclerotized part of the pupal cuticle, is generally similar to that of tanned cuticle, except that the level of catechols is increased fourfold [Fig. 7(c), line No. 3, 144 ppm]. These results support the hypothesis that insect cuticle is sclerotized by incorporation of catechols into the protein-chitin-lipid composite of newly ecdysed soft cuticle.

The chemical composition of adult cuticles from six coleopteran species was estimated by solids NMR (Kramer *et al.*, 1989b). The adult cuticles are dark brown in color and similar in appearance to *M. sexta* pupal cuticle. Of all cuticles examined to date, adult beetle cuticles contain the highest percentage of catechols, ranging from 16–33% (Table 5). In addition to protein, chitin, catechols and lipid, the presence of the pigment melanin in the exoskeleton is confirmed by solids NMR analysis of elytra from wild type and *black* mutant strains of the red flour beetle, *Tribolium castaneum* (Fig. 8). The  $^{13}\text{C}$  NMR difference spectrum obtained by subtracting the spectrum of the wild type from that of the *black* mutant strain shows that both elytra have comparable levels of protein, chitin, and lipid, but that the *black* elytra have more carbon signals characteristic of melanin (positive carbon resonances at 0–30 ppm and 80–160 ppm). In addition, wild type elytra have more carbon signals characteristic of  $\beta$ -alanine (negative carbon resonances at 35 and 175 ppm). These results are in agreement with results obtained by wet chemical analysis, which reveal that the *black* mutant has higher levels of dopamine, a melanin precursor, and lower levels of  $\beta$ -alanine and *N*- $\beta$ -alanyldopamine relative to the wild type strain (Kramer *et al.*, 1984; Roseland *et al.*, 1987). These chemical differences lead to production of melanin when the excess dopamine in the *black* strain is oxidized.

TABLE 5. Relative percentages of organic components in cuticles and exuviae from beetles determined by solids NMR analysis\*

Species	Relative amount (%)			
	Protein	Chitin	Catechol	Lipid
<i>Sitophilus oryzae</i> adult	33	33	32	2
<i>S. granarius</i> adult	32	36	30	2
<i>S. zeamais</i> adult	30	35	33	2
<i>Tenebrio molitor</i>				
Elytra	42	41	16	1
Hindwing	61	24	14	1
Larval exuviae	82	11	6	1
Pupal exuviae	67	25	6	2
Larval abdomen	76	16	7	1
<i>Tribolium castaneum</i> adult				
Wild type	38	33	25	4
Black	37	32	27	4
<i>T. confusum</i> adult	38	33	24	5

\*Determined by  $^{13}\text{C}$  NMR. Unit = percentage of total carbon. From Kramer *et al.*, 1989b.

Another species that offers a comparison of mutant cuticular phenotypes for NMR analysis is the Mediterranean fruit fly, *Ceratitis capitata*. In the mutant *white pupa*, the puparium fails to tan, but normally tanned larval and adult cuticular structures develop. The mutant puparium is fivefold less resistant to compression than the wild type strain (Wappner *et al.*, 1995). Solid state  $^{13}\text{C}$  NMR analysis reveals that more chitin, but less  $\beta$ -alanine and fewer catechols are present in the mutant's

cuticle relative to the wild type cuticle. Apparently, the *white pupa* mutant is defective in a transport mechanism for providing catecholamines to the puparial cuticle, thus preventing normal sclerotization and pigmentation (Wappner *et al.*, 1995).

Two dipteran species were used to compare sclerotized and mineralized cuticles using solids NMR analysis. The puparial cuticle of the house fly, *Musca domestica*, is stabilized primarily by sclerotization, in which catecholic

## RED FLOUR BEETLE

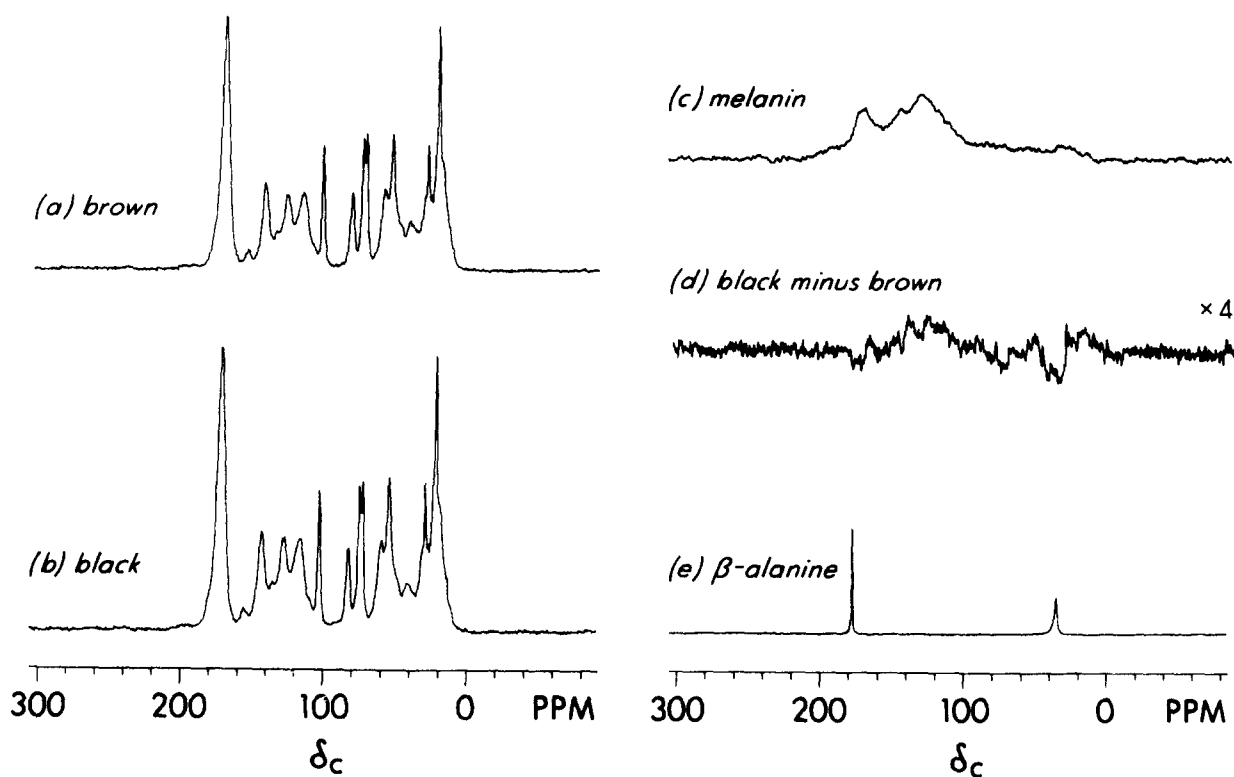


FIGURE 8.  $^{13}\text{C}$  CPMAS NMR spectra of *Tribolium castaneum* cuticle. (a) Elytra from wild type strain; (b) elytra from black strain; (c) dopa melanin standard; (d) difference spectrum, black minus wild type; and (e)  $\beta$ -alanine standard. From Kramer *et al.* (1989b). See Table 1 for chemical shift assignments.

TABLE 6. Chemical composition of the puparial exuviae from the house fly, *Musca domestica*, and the face fly, *M. autumnalis* as determined by solids NMR analysis\*

Component	House fly	Face fly	Ratio (HF:FF)
Chitin	45	19	2.4
Protein	31	10	3.1
Catechol	13	1	13.0
Lipid	2	2	1.0
Water	6	5	1.2
Minerals	3	63	0.05

\*From Kramer *et al.*, 1988. Unit = percentage of wet weight. Protein, chitin, catechol, and lipid determined by  $^{13}\text{C}$  NMR; water and minerals (ash content) by gravimetric analysis.

metabolites accumulate in, and presumably cross-link, the protein/chitin matrix of the larval cuticle (Roseland *et al.*, 1985). The face fly, *M. autumnalis*, on the other hand, hardens its puparial cuticle by depositing calcium and magnesium phosphates into the larval cuticle. Whereas mineral salts constitute more than 60% of the face fly puparial exuviae, they make up only 3% of the house fly puparial exuviae (Table 6; Kramer *et al.*, 1988). House fly cuticle contains substantially more protein, chitin and catecholic compounds than does face fly cuticle. Protein, chitin and catechols make up approx 90% of the house fly cuticle, whereas they account for less than 30% of the face fly cuticle. The results demonstrate that dipterans use both catecholamines and minerals for stabilization of puparial cuticle, with the house

fly relying primarily on sclerotization and the face fly on mineralization.

#### Heteronuclear interactions

Intermolecular cross-linking of proteins is considered to be one of the primary mechanisms for sclerotization of insect cuticles, silk structures, egg shells and egg cases, but because of the intractable nature of sclerotized structures, little direct evidence for a cross-link structure has been reported (Hopkins and Kramer, 1992). Solids NMR has been extremely useful for probing the cross-link structures in the pupal cuticle of the tobacco hornworm, and the data support the cuticle model originally proposed by Pryor (1940a, b), which depicts protein chains cross-linked by quinonoid derivatives of catechols. NMR analyses of *M. sexta* pupal cuticle containing protein labeled with 1,3- $^{15}\text{N}_2$  histidine demonstrates that a side chain histidyl becomes attached to a carbon atom during sclerotization (Schaefer *et al.*, 1987; Christensen *et al.*, 1991b). After the pupal cuticle is doubly labeled with both 1,3- $^{15}\text{N}_2$  histidine and ring- $^{13}\text{C}_6$  dopamine, the double cross-polarization spectrum reveals that one of the aromatic catecholamine carbon atoms is bonded covalently to a ring nitrogen of histidine (Fig. 9). The CPMAS  $^{15}\text{N}$  NMR spectral data of pupal cuticle labeled by injection of L-1,3- $^{15}\text{N}_2$  histidine also support the formation of carbon-nitrogen cross-links. The  $^{15}\text{N}$  NMR spectrum of unsclerotized cuticle shows

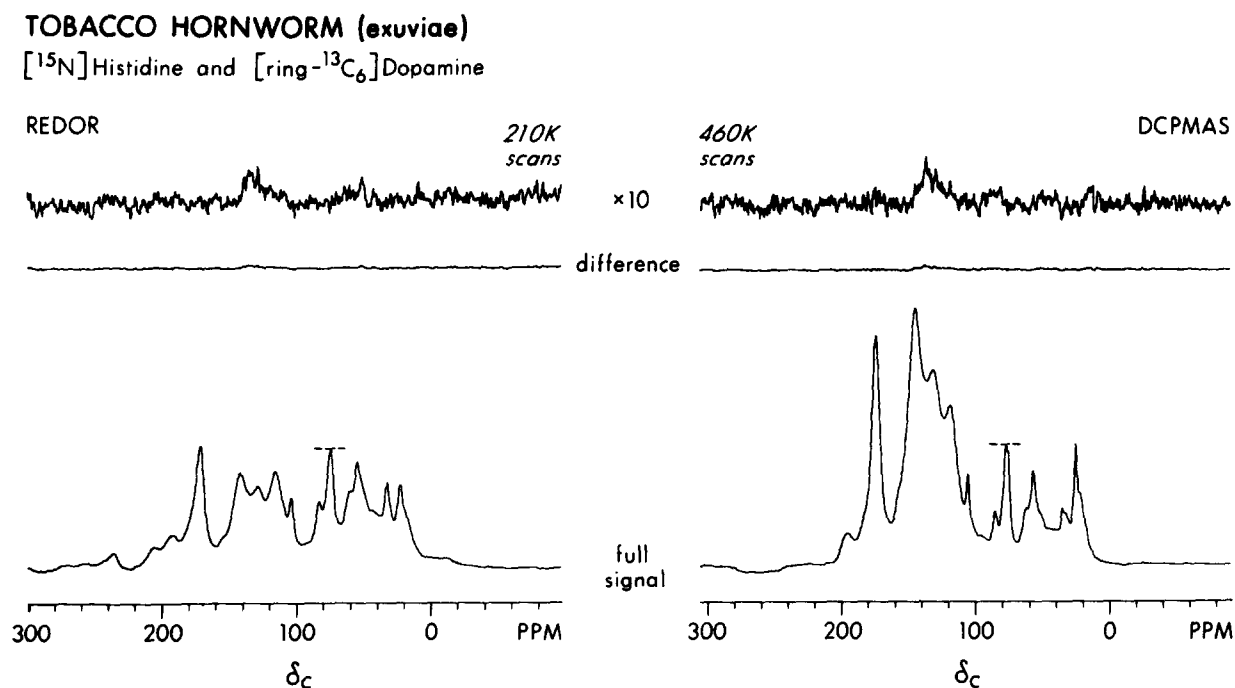


FIGURE 9. REDOR (left) and DCP (right) full signal (bottom) and difference (middle =  $1 \times$ , top =  $10 \times$ ) magic angle spinning  $^{13}\text{C}$  NMR spectra of tobacco hornworm pupal exuviae labeled with L-[ring- $^{15}\text{N}_2$ ] histidine and [ring- $^{13}\text{C}_6$ ] dopamine. Spectra were obtained at 50.3 MHz with magic angle spinning at 3.205 kHz. REDOR dephasing was summed over four rotor cycles with  $^{15}\text{N}$   $\pi$  pulses every half rotor period. DCP spectra were obtained with total suppression of spinning sidebands following a 3 ms carbon-nitrogen spin lock. The natural abundance chitin peaks at 80 ppm indicated by the dashed lines are equal in intensity. About half of the 140 ppm signal in the REDOR difference spectrum and one quarter of the 140 ppm DCP difference signal are due to natural abundance  $^{13}\text{C}$  in the histidine ring. From Christensen *et al.* (1991b). See Table 1 for chemical shift assignments.

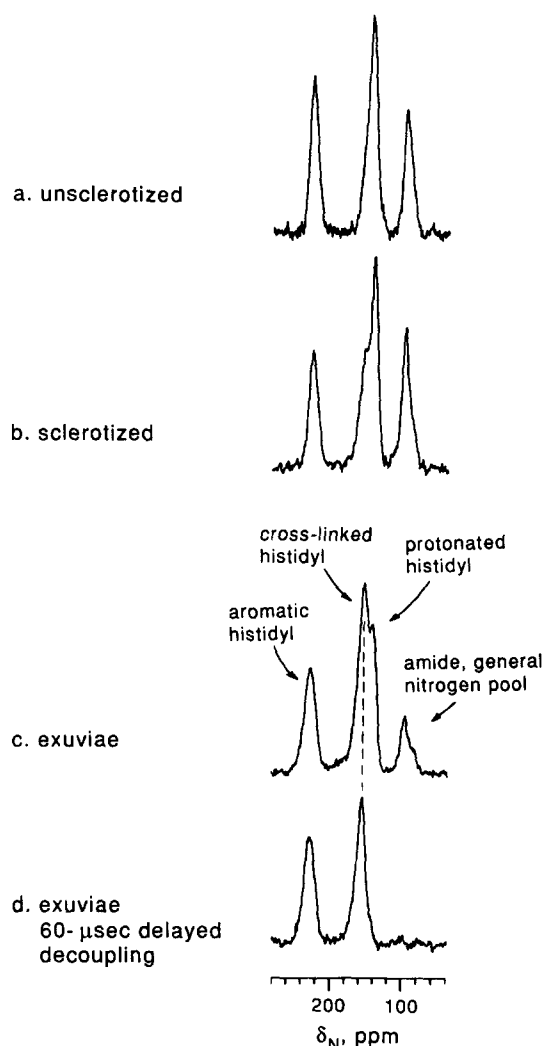


FIGURE 10. CPMAS  $^{15}\text{N}$  NMR spectra of L-[1,3- $^{15}\text{N}_2$ ] histidine-labeled (a) newly ecdysed unsclerotized pupal cuticle, (b) 3-day-old sclerotized pupal cuticle, (c) pupal exuviae, and (d) pupal exuviae under delayed decoupling. Spectra were acquired at 20.3 MHz through the use of matched spin lock cross polarization transfers with 1 ms contacts and 35 kHz radio frequency fields. Only nonprotonated nitrogen resonances survive delayed decoupling. The vertical display for the top two spectra is approximately twice that of the bottom two. The horizontal scale is in part per million downfield from solid ammonium sulfate as an external reference. From Schaefer *et al.* (1987).

protonated and nonprotonated histidyl ring nitrogen peaks at 140 and 225 ppm, respectively (Fig. 10), as well as a natural abundance amide nitrogen peak at 100 ppm. In 3-day-old sclerotized cuticle, a new signal is observed at 155 ppm that builds up during the time course of sclerotization and becomes a major nitrogen resonance in the exuviae. Dipolar dephasing produced by delayed  $^1\text{H}$  decoupling has little effect on the new signal, which shows the nitrogen to be nonprotonated. Its chemical shift indicates that a histidyl nitrogen is attached to either an aromatic or aliphatic carbon. Taken together, the  $^{13}\text{C}$  and  $^{15}\text{N}$  NMR data are consistent with the formation of an aromatic carbon–nitrogen bond via a mechanism whereby an imidazole nitrogen attacks a phenyl carbon of an *o*-quinone derivative of the catecholic compound.

Schaefer *et al.* (1987) also proposed that covalent bonds exist between cuticular protein and chitin. Solid state  $^{15}\text{N}$  NMR analysis of chitin prepared by alkali extraction of 1,3-[ $^{15}\text{N}_2$ ]-histidine-labeled *M. sexta* pupal exuviae revealed an  $^{15}\text{N}$  chemical shift expected for the substituted imidazole nitrogen cross-link structure. Apparently, the chitin is not coupled directly to protein, but instead to a catecholic carbon, which serves as a bridge between protein and chitin. Because the evidence for a protein–chitin linkage in cuticle is only of an indirect nature, additional work is needed to obtain more substantive direct evidence for such a linkage.

Cross-links between a histidyl ring nitrogen and the  $\beta$ -carbon of dopamine have been postulated to occur in insect cuticle as a result of  $\beta$ -sclerotization or quinone methide sclerotization (Andersen, 1985; Sugumaran, 1988). However, double cross-polarization NMR spectroscopy is too insensitive for detecting this type of linkage because of inherently slow C–N polarization transfer and fast spin-lock relaxation for the labeled dopamine carbon (Kramer and Hopkins, 1987; Schaefer *et al.*, 1987). REDOR circumvents these technical problems, provides an order of magnitude improvement in sensitivity, and was used to identify covalent bond formation between the  $\beta$ -carbon of dopamine and ring nitrogen of histidine (Christensen *et al.*, 1991a). In cuticle labeled by both  $\beta$ - $^{13}\text{C}$  dopamine and ring [ $^{15}\text{N}_2$ ]histidine, only the resonance at  $\delta_{\text{C}} 60$  has a  $^{13}\text{C}$  REDOR difference signal above the  $^{15}\text{N}$  natural abundance level (Fig. 11). This signal arises from the formation of a protein–catecholamine  $\beta$ -carbon covalent bond. The weak difference signal at 120 ppm is due to natural abundance carbons in  $^{15}\text{N}$  labeled histidine. We estimate that about two-thirds of the bound histidyl residues in the exocuticular protein are linked covalently to catecholamine ring carbons, and about one-third are linked to the  $\beta$ -carbon in pupal cuticle. NMR experiments with double-labeled  $^{13}\text{C}$ - $^{15}\text{N}$ - $\beta$ -alanine indicate that the terminal amino group of *N*- $\beta$ -alanyldopamine also is involved in covalent bonding. Use of dopamine labeled with  $^{13}\text{C}$  in the  $\alpha$ -position of the side chain provides evidence of covalent bonding to the  $\alpha$ -carbon, but the nature of the respective adducts is not established. The formation of oligomeric forms of catecholamines may occur through the  $\alpha$ -carbon,  $\beta$ -carbon, and/or ring addition reactions with quinonoid compounds, resulting in larger cross-linking agents and space fillers in the cuticular matrix (Andersen *et al.*, 1992a, b). Although solids NMR has provided good evidence for portions of the cross-link structure of the insect cuticle, the supramolecular structure is still incomplete and little information has been obtained about the nature of the transient intermediates that serve as cross-linking agents. Solids NMR data are consistent with the hypothesis that quinones and quinone methides serve as cross-linking agents, but other intermediates are also possible.

Solids NMR analysis provides direct evidence for covalent modifications to the ring carbons, the  $\beta$ -



*et al.*, 1994), will be utilized to detect single and multiple heteronuclear connectivities in cuticle.

The results of a preliminary experiment to examine *N*- $\beta$ -alanyldopamine chemistry in *M. sexta* pupal cuticle labeled by  $\beta$ - $^{15}\text{N}$ -alanine and  $\alpha$ - $^{13}\text{C}$ -dopamine reveal a  $^{13}\text{C}$  REDOR difference spectrum with four distinct peaks at 175, 170, 70, and 40 ppm (Schaefer *et al.*, unpublished data). The most surprising result is the 70 ppm REDOR difference peak, which suggests a double nitrogen substitution of the labeled dopamine carbon. This kind of chemistry could be achieved by an *N*- $\beta$ -alanyldopamine ring closure, resulting in a structure with nitrogens functionally capable of covalently linking to other catechols, proteins, or chitin, or by condensation of two different catechol molecules. This intramolecular cyclization would link the  $^{13}\text{C}$  label of dopamine with the  $^{15}\text{N}$  label of  $\beta$ -alanine, producing the 70 ppm REDOR difference signal. We can test for the occurrence of a ring closure by labeling cuticle with  $\alpha$   $^{13}\text{C}$ ,  $^{15}\text{N}$  dopamine. This sample would have high levels of the 70 ppm REDOR difference peak, assuming ring closure. In addition, by labeling with both  $\alpha$   $^{13}\text{C}$ ,  $^{15}\text{N}$  dopamine and  $\beta$ - $^{15}\text{N}$ -alanine, we may be able to create sizeable concentrations of three spin labels and detect them by differences in REDOR and TEDOR rates for  $^{13}\text{C}$  observation compared to  $^{15}\text{N}$  observation. TEDOR is a rotor synchronized solid-state experiment that selects dipolar coupled spins from among the background of uncoupled spins by a coherence transfer from one spin of the heteronuclear pair to the other (Hing *et al.*, 1992). Triplets like  $^{13}\text{C}$ — $^{15}\text{N}$ — $^{13}\text{C}$ —,  $^{15}\text{N}$ — $^{13}\text{C}$ — $^{15}\text{N}$ —, and  $^{13}\text{C}$ — $^{17}\text{O}$ — $^{13}\text{C}$ — can be detected specifically by double coherence transfers.

Additional solids NMR experiments will be designed to detect carbon-oxygen and carbon-carbon cross-links in the *Manduca* exoskeleton. No direct evidence for C—O or C—C linkages between protein, chitin, and catecholamines has been reported to date. However, residual protein and catecholamines are detected in chitin extracted from hornworm pupal cuticles (Schaefer *et al.*, 1987), suggesting the presence of C—O cross-links to chitin. Cuticular samples labeled with either 3,4- $^{17}\text{O}_2$ -dopamine or  $^{17}\text{O}_5$ -N-acetylglucosamine and either  $^{13}\text{C}$ -labeled amino acids, catecholamines, or *N*-acetylglucosamine will be examined for the presence of  $^{13}\text{C}$ — $^{17}\text{O}$  linkages between catecholamines, proteins, and chitin. A REDOR-based method to monitor indirectly the chemical connectivity of quadrupolar nuclei such as deuterium and  $^{17}\text{O}$  has been developed recently at Washington University and will be utilized for  $^{17}\text{O}$ -labeled cuticle samples. Another NMR technique being developed for measuring  $^{13}\text{C}$ — $^{13}\text{C}$  distances is similar to the REDOR method and is called combined-rotation with nutation (CROWN) NMR (Joers *et al.*, 1994). The power and specificity of these and other novel solid state NMR techniques will allow direct testing of hypotheses about insect structural biochemistry, which has not been possible in the past.

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